INTRACELLULAR OSMOREGULATION IN SKELETAL MUSCLE DURING SALINITY ADAPTATION IN TWO SPECIES OF TOADS [1]

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The European green toad (*Bufo viridis*) is a surprisingly euryhaline amphibian, some populations living in environmental salinities as high as 29‰ (Stoicovici and Pora, 1951). This survival appears to be largely based upon a tolerance by the tissues of body fluid osmotic concentrations somewhat higher than those of the environment. Changes in osmotic concentrations of body fluids are due primarily (up to 84%) to changes in NaCl content (Gordon, 1962; Tercafs and Schoffeniels, 1962).

The occurrence of large changes in osmotic and salt concentrations in extracellular fluids immediately raises the question of the nature of possible simultaneous changes in intracellular fluid composition. The present paper describes the results of a study of the changes in the principal intracellular solutes in the skeletal muscles of adult green toads adapted to various environmental salinities. For comparison, similar data are presented for the less euryhaline western American toad, *Bufo boreas*. A preliminary account of some of the results of this work was given by Gordon (1963).

Materials and Methods

Adult green toads were collected on the island of Saltholm, near Copenhagen, Denmark, in May, 1962, and were shipped by air to Los Angeles. In Los Angeles they were maintained at 19 ±2° C. in tap water dechlorinated with a charcoal filter. They were fed twice weekly with larval and adult *Tenebrio*. Survival was excellent. Some toads not used in experiments remained alive and in good condition for more than two years. No attempt was made to separate the sexes.

Adult western toads (*Bufo boreas*) were obtained as needed from local suppliers in southern California. Maintenance conditions were as for green toads.

Toads were adapted for two or more days to dilutions of natural sea water in groups of six or more in an air-conditioned room maintained at 19 ±2° C. Sea water dilutions (100% sea water ≅ 32‰ salinity = 950 mOsm/l. osmotic concentration) were made with dechlorinated tap water. Higher salinities were ap-

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TABLE I

Analytical procedures

Analysis	Procedure	Plasma sample vol. (μl.)	Average precision of dupl. analyses	Muscle sample wt. (mg.)	Average precision of dupl. analyses	Reference
Osmolarity	Freezing point depression	0.001	±20 mOsm/l.	—		Ramsay & Brown (1955)
Total solids	Wet-dry weights	—	—	100–200	±2 gm. kg.	Gordon (1962)
Chloride	Ag^+ titration	50	± 3mEq l.	100–200	±1 mEq/kg.	Cotlove (1963)
Sodium	Flame photometer	100	±3 mEq/l.	100–200	±0.5 mEq/kg.	Gordon (1962); for muscle, water homogenates of fresh samples.
Potassium	Flame photometer	100	±0.2 mEq/l.	100–200	±2 mEq/kg.	Gordon (1962); for muscle, water homogenates of fresh samples.
Non-protein N (NPN)	Nesslerization	50	±20 mg. l.	100–200	±0.03 gm./kg.	Natelson (1961)
α-NH_2N	Na β-naphthoquinone-4-sulfonate	100–200	±2 mg./l.	100–200	±0.02 gm./kg.	Frame *et al.* (1943); Russell (1944)
Urea	Urease, micro-diffusion	25	± 10 mg./l.	100–200	±0.01 gm./kg.	Conway (1957a); for muscle as for sodium above
Inulin	Resorcinol	100	±30 mg./l.	100–200	±0.001 gm. kg.	Roe *et al.* (1949); see text
Total sugar	Anthrone	—	—	50–70	±0.2 gm./kg.	Seifter & Dayton (1950)
Glycogen	Anthrone	—	—	50–70	±0.2 gm./kg.	Seifter & Dayton (1950)
Free amino acids (qualitative)	2-dimensional paper chromatography	100	—			Allen & Awapara (1960); Scherbaum *et al.* (1959); see text
Free amino acids (quantitative)	Spinco Model 120 amino acid analyzer	—	—	1000–1500	see text	Spackman *et al.* (1958)

proached in graded steps (*e.g.*, successive three-day periods in fresh water, 20%, 40% and 50% sea water). Toads were not fed during salinity adaptation.

Blood samples were obtained by exposing and opening the heart, then collecting the blood in lightly heparinized glass capillary tubes. These tubes were closed with

TABLE II

Inulin spaces in toads adapted to various salinities

State of adaptation	[$\bar{x}$ ± S.E.(N)]	
	Total extracellular fluid (l./kg. body wt.)	Muscle extracellular fluid (l./kg. wet wt.)
Bufo viridis		
FW	0.418 ± 0.019 (5)	0.128 ± 0.036 (5)
20% SW, 7–15 days	0.398 ± 0.019 (4)	0.139 ± 0.035 (3)
40% SW, 5–13 days	0.364 ± 0.014 (5)	0.132 ± 0.060 (4)
50% SW, 2–6 days	0.338 ± 0.022 (5)	0.175 ± 0.011 (5)
Bufo boreas		
FW, start	0.332 ± 0.026 (4)	0.116 ± 0.035 (5)
FW, starved 1 mon.	0.331 ± 0.019 (3)	0.120 ± 0.072 (3)
20% SW, 7–15 days	0.365 ± 0.020 (5)	0.139 ± 0.038 (3)
40% SW, 2–8 days	0.288 ± 0.037 (5)	0.164 ± 0.034 (5)
50% SW, 3 days	0.270 (1)	0.159 (1)

sealing wax, centrifuged and resealed after the packed red cells were cut away. Plasma samples were stored frozen at −20° C. for later analysis.

Samples of skeletal muscle ranging in weight, as required by the experiment, from 50 to more than 1000 mg. were taken from the thighs. Muscle samples were subdivided and the pieces weighed to ±0.2 mg. on a torsion balance. Prepared muscle extracts were stored frozen at −20° C. for later analysis.

Procedures for sample preparation and analysis are summarized in Table I. Some additional information is necessary for two procedures.

Inulin spaces: Inulin spaces ("extracellular spaces") were determined both for the whole animal and for thigh musculature. Toads were weighed to ±0.1 g., then injected intraperitoneally with 0.5 ml./100 g. of 10% inulin in 0.5% NaCl solution. Pilot studies on both species showed that plasma inulin concentrations reached a plateau stable for at least four hours within two hours of injection.

TABLE III

Plasma concentrations in toads adapted to various salinities
[$\bar{x}$ ± S.E. (N)]

State of adaptation	Osmolarity (mOsm l.)	Cl^- (mEq l.)	Na^+ (mEq l.)	K^+ (mEq l.)	NPN (mg. l.)	Free $\alpha-NH_2N$ (mg. l.)	Urea N (mg. l.)
Bufo viridis							
FW	279±13(5)	99±2(5)	113±4(5)	4.7±0.4(5)	1040±110(5)	46±4(10)	970±150(5)
20% SW 7–15 days	290±10(5)	114±3(5)	126±2(5)	5.8±0.3(5)	920±40(5)	45±3(10)	790±40(5)
40% SW 5–13 days	441±8(5)	159±5(5)	173±4(5)	6.5±0.3(5)	1980±130(5)	70±6(12)	1930±150(4)
50% SW 2–6 days	502±9(5)	196±1(5)	207±2(5)	10.0±0.8(5)	2090±60(4)	78±5(9)	1920±60(5)
Bufo boreas							
FW start	156±22(5)	61±8(5)	64±25(4)	4.1±0.2(5)	580±110(5)	58±6(9)	630±160(4)
FW starved 1 mon.	—	—	—	—	—	39±3(6)	—
20% SW 7–15 days	253±15(4)	102±2(5)	98±11(5)	4.8±0.2(5)	590±60(5)	57±3(10)	420±50(5)
40% SW 2–8 days	366±15(3)	172±1(4)	183±2(3)	5.9±0.1(3)	1150±40(3)	88±8(10)	850±130(4)
50% SW 3–6 days	—	—	—	—	—	84±6(3)	—

Plasma and muscle samples were taken from injected toads 2–6 hours after injection. Paired control samples were taken from similarly treated uninjected animals. Following analysis, mean background values for inuloid substances in the muscles were subtracted from the measured inulin values in injected toads. This background was usually quite constant within groups treated in a given way. No background correction was necessary for plasma. There was always close agreement between the muscle inulin spaces measured in the two thighs of individual animals.

Muscle free α-NH_2N and free amino acids: Muscle samples for these analyses were taken immediately after sacrifice of the experimental animal. They were weighed on a torsion balance and homogenized in ice-cold 10% trichloroacetic acid (for α-NH_2N), 80% ethanol (for paper chromatography) or 1% picric acid (for the amino acid analyzer). Checks on possible rapid production of amino acids by hydrolysis of tissue proteins were made for the first two methods by doing

TABLE IV

Muscle concentrations in toads adapted to various salinities

[All amounts per kg. wet wt.; $\bar{X} \pm$ S.E.(N)]

State of adaptation	Total solids (gm.)	Cl^- (mEq)	Na^+ (mEq)	K^+ (mEq)	NPN (gm.)	Free α-NH_2N (gm.)	Urea N (gm.)	Total sugar (gm.)	Glycogen (gm.)
Bufo viridis									
FW	210±4(10)	24±1(5)	34±1(5)	64±1(5)	3.7±0.2(5)	0.94±0.02(10)	0.83±0.15(5)	10.1±0.7(5)	9.6±0.9(5)
20% SW 7–15 days	211±5(10)	27±2(5)	32±2(5)	65±2(5)	3.3±0.1(5)	1.00±0.03(10)	0.88±0.06(5)	—	—
40% SW 5–13 days	249±5(12)	57±4(5)	68±4(5)	66±2(5)	4.6±0.1(5)	1.31±0.06(12)	1.82±0.18(5)	7.3±1.1(5)	6.7±1.3(5)
50% SW 2–6 days	281±4(9)	70±4(5)	78±5(5)	72±2(5)	4.9±0.1(5)	1.48±0.06(9)	1.76±0.09(5)	—	—
Bufo boreas									
FW start	208±5(9)	20±2(5)	27±2(5)	75±6(5)	2.8±0.3(5)	0.71±0.05(9)	0.68±0.14(5)	7.8±0.4(5)	7.2±0.4(5)
FW starved 1 mon.	210±3(6)	—	—	—	—	0.70±0.03(6)	—	—	—
20% SW 7–15 days	194±2(10)	27±3(5)	37±3(4)	77±3(5)	3.0±0.1(5)	0.85±0.02(9)	0.56±0.06(5)	—	—
40% SW 2–8 days	270±6(10)	49±7(4)	62±8(4)	86±11(4)	3.8±0.1(4)	1.22±0.04(10)	1.15±0.09(4)	10.5±1.0(5)	9.5±1.0(5)
50% SW 3–6 days	276±8(3)	—	—	—	—	1.39±0.09(3)	—	—	—

parallel analyses on duplicate aliquots of tissue which were either allowed to remain at room temperature for several minutes before being homogenized or were homogenized in the appropriate solutions at room, rather than ice, temperatures. No statistically significant differences were found. These results agree with those of Roberts and Simonsen (1962).

Mean intracellular concentrations for substances in the muscles were calculated using the following equation:

$$[A]_i = \frac{[A]_m - [A]_p v_e}{[H_2O]_m - v_e}$$

$[A]_i$ = mean intracellular concentration of A
$[A]_m$ = mean wet muscle concentration of A
$[A]_p$ = mean plasma concentration of A
v_e = mean muscle inulin space
$[H_2O]_m$ = mean wet muscle water content

The most variable quantity involved in these calculations is v_e. Estimates of the variability in $[A]_i$ produced by variation in v_e were obtained by recalculating $[A]_i$ using $v_e \pm 2$ S.E.'s. The variability figures given in Table V are the average deviations about the means, calculated by this procedure.

These calculations were made on the assumption that there are no large differences between plasma concentrations and concentrations in muscle extracellular fluid. The data of Conway (1957b) and Maurer (1938) support this assumption

TABLE V

Intracellular concentrations in muscles of toads adapted to various salinities (all amounts per kg. cell water; cf. text for method of calculation)

State of adaptation	Cl^- (mEq)	Na^+ (mEq)	K^+ (mEq)	NPN (gm.)	Free α-NH_2N (gm.)	Urea N (gm.)
Bufo viridis						
FW	17 ± 8	30 ± 10	95 ± 10	5.4 ± 0.5	1.42 ± 0.01	1.07 ± 0.01
20% SW 7–15 days	17 ± 10	22 ± 11	98 ± 10	4.9 ± 0.4	1.52 ± 0.01	1.18 ± 0.04
40% SW 5–13 days	58 ± 20	73 ± 20	105 ± 20	7.0 ± 1.0	2.10 ± 0.02	2.54 ± 0.11
50% SW 2–6 days	66 ± 6	77 ± 5	130 ± 6	8.4 ± 0.3	2.68 ± 0.16	2.61 ± 0.02
Bufo boreas						
FW start	19 ± 4	30 ± 4	110 ± 10	4.0 ± 0.4	1.04 ± 0.20	0.90 ± 0.02
FW starved 1 mon.	—	—	—	—	1.03 ± 0.02	—
20% SW 7–15 days	19 ± 10	34 ± 8	114 ± 12	4.3 ± 0.4	1.26 ± 0.01	0.75 ± 0.04
40% SW 2–8 days	37 ± 16	57 ± 15	150 ± 18	6.4 ± 0.6	2.14 ± 0.03	1.78 ± 0.12
50% SW 3–6 days					2.44 ± 0.05	

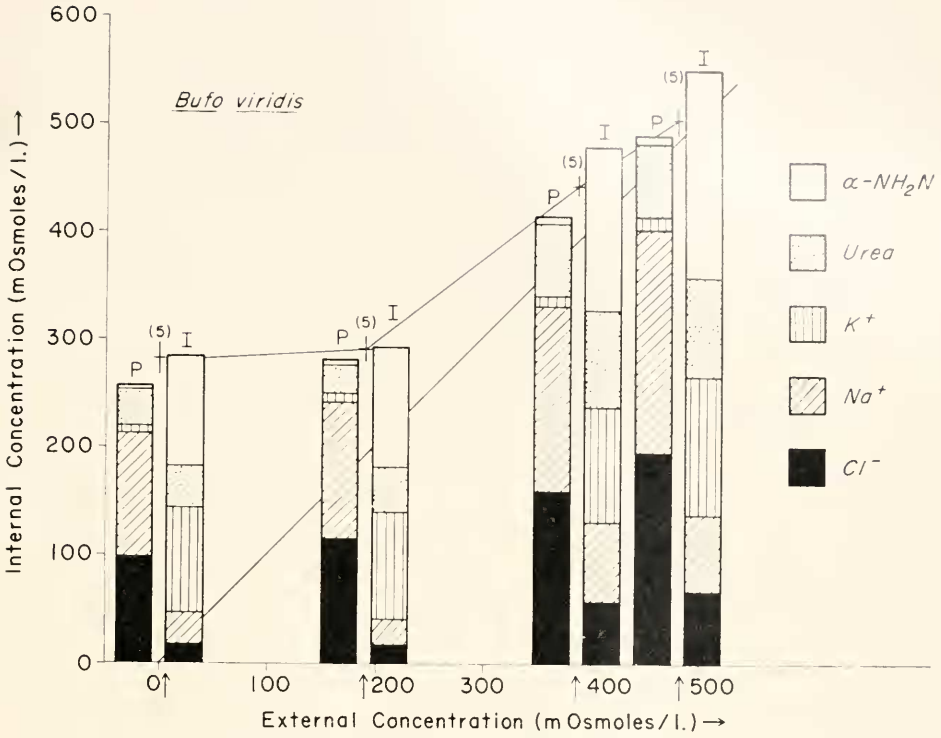

FIGURE 1. Composition of plasma (P) and intracellular fluid (I) in skeletal muscles of *Bufo viridis* acclimatized to fresh water, 20%, 40% and 50% sea water (arrows along abscissa indicate acclimatization concentrations). Data from Tables III and V. Upper continuous line, plasma osmotic concentration; second continuous line, equality between internal and external concentrations. Vertical lines on each point ±2 S.E.'s of means. Left-hand bar in each pair plasma concentrations; right-hand bar calculated intracellular concentrations.

for frogs. Sodium concentrations were not corrected for possible sarcolemmal binding (Conway and Carey, 1955; Dee and Kernan, 1963).

RESULTS

Whole body and muscle inulin spaces in both *Bufo viridis* and *Bufo boreas* adapted to various concentrations of sea water are listed in Table II. There are differences between the two species with respect to whole body space, but no such differences for muscle space. *B. viridis* had whole body inulin spaces 3–8% larger than those found in similarly adapted *B. boreas*. Neither salinity adaptation nor starvation for a month significantly affected whole body spaces in *B. boreas*. In *B. viridis*, however, whole body space decreased by 7% (significant at 5% level by "t" test) between fresh water and 50% sea water.

The maximum environmental concentration tolerated indefinitely by *B. viridis* was 50% sea water. *B. boreas* survived well in 40% sea water, but died within a week in 50% sea water. Whole animal and muscle inulin spaces in one *B.*

boreas, after three days in 50% sea water, were insignificantly different from those of normal-appearing, active toads in 40% sea water.

Muscle inulin spaces in both species are in the range usually reported for amphibian skeletal muscle. There were no statistically significant variations in muscle inulin spaces in differently adapted animals in either species. Variability between animals was fairly large, however, so possible small differences may be masked.

The results of analyses of plasma and muscle samples taken from variously adapted *B. viridis* and *B. boreas* are presented in Tables III and IV. These basic data have been combined with the muscle inulin space data of Table II to yield estimates of intracellular concentrations in the skeletal muscle fibers of the two toads. These latter estimates are presented in Table V. Figures 1 and 2 compare the major features of these sets of data.

Both forms maintain osmotic concentrations of the plasma at levels higher than medium concentrations until maximum salinity tolerances are reached. Increases in plasma concentrations above those characteristic of fresh water are due primarily to NaCl (86% of the increase in *B. viridis* between fresh water and 50% sea water; 109% of the increase in *B. boreas* between fresh water and 40% sea water—

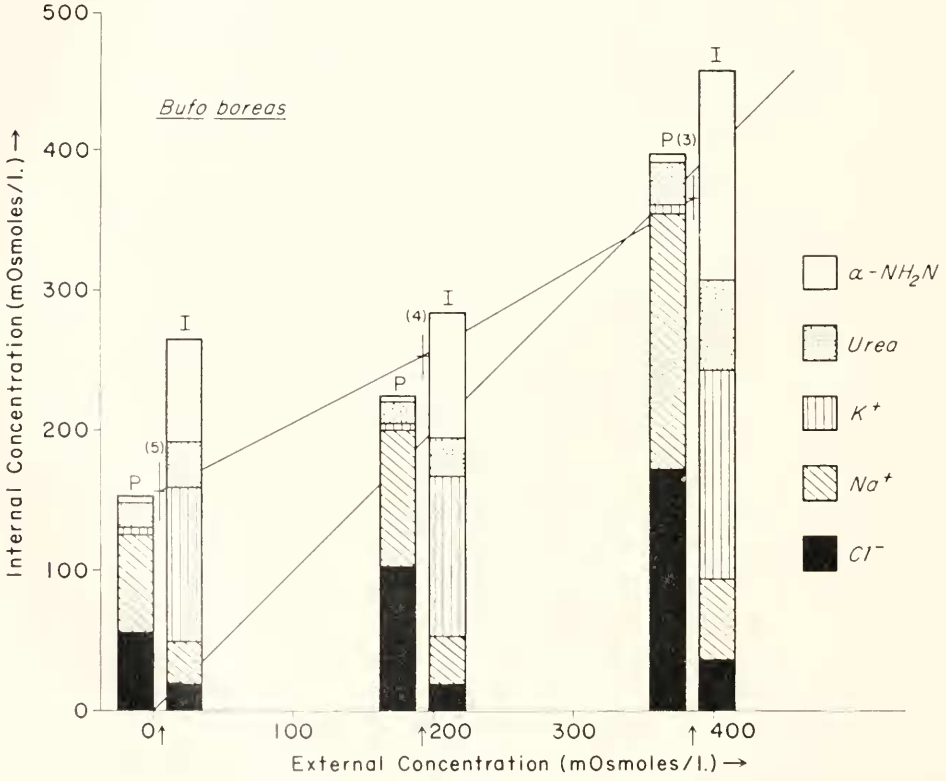

FIGURE 2. As Figure 1 but for *Bufo boreas*. Toads acclimatized to fresh water, 20% and 40% sea water.

a mathematical anomaly due primarily to the variability of the osmolarity measurements). Changes in plasma urea concentrations account for only a small fraction of the osmolarity changes (14% in *B. viridis* between fresh water and 50% sea water; 4% in *B. boreas* between fresh water and 40% sea water). Plasma amino acid concentrations are low (3–6 m*M*) in both forms in all salinities. In all salinities plasma urea N plus α-NH_2N account for essentially all plasma NPN in *B. viridis* and for 81% or more of plasma NPN in *B. boreas*.

Plasma osmolarity increased 80% in *B. viridis* between fresh water and 50% sea water, and 135% in *B. boreas* between fresh water and 40% sea water. Muscle solids in the same groups of animals, however, increased by only 33% in both species. The muscles, therefore, maintained great stability in water content in the face of major changes in extracellular osmotic concentration. Since muscle inulin spaces were constant, there were only minor decreases in fiber volumes. The main phenomenon was adjustment of intracellular concentrations so as to minimize hydration changes.

The calculated intracellular concentrations indicate that all measured cellular solutes contributed to these adjustments. The major contributions in both forms came from the inorganic ions, urea, and free amino acids and related compounds. No significant amounts of free carbohydrates were present in the muscles of either species.

Rough estimates of total intracellular osmotic concentrations may be obtained from the data in Table V by calculating the millimolar equivalents for α-NH_2N and urea N, then adding these to the inorganic ion data. One may then calculate the fractional contributions made to changes in total estimated intracellular osmotic concentrations by changes in the various components.

For *B. viridis* between fresh water and 50% sea water, chloride and sodium each accounted for 17%, potassium for 13% of the estimated total change. These three ions together accounted for about 47% of the change. Free amino acids accounted for about 33%, urea for the remaining 20% of the estimated change.

For *B. boreas* between fresh water and 40% sea water, chloride and sodium, respectively, accounted for 9% and 14%, potassium for 20% of the total intracellular change. These ions together accounted for 43% of the change. Free amino acids comprised 40%, urea 17% of the estimated difference.

In all states of salinity adaptation, intracellular urea concentrations in both species apparently exceeded plasma urea levels. The ratio $urea_{in}/urea_{out}$ was variable, ranging from 1.10 to 1.50 in *B. viridis*, from 1.42 to 2.10 in *B. boreas*. If intracellular urea is not bound, as is usually assumed (Bozler, 1961), these ratios would indicate significant urea accumulation by toad muscle fibers.

Urea and amino acids accounted for almost all of the changes in intracellular NPN which occurred. Starvation for one month produced no change in intracellular free α-NH_2N in *B. boreas*.

The finding of large changes in muscle content of free amino acids raised the question of which amino acids were involved. Muscle samples were taken from five toads of each species adapted to fresh water and from five toads of each species adapted to 40% sea water. Separate one- and two-dimensional paper chromatograms were run of acidic and basic amino acid fractions from each sample. These chromatograms demonstrated: (1) Major amino acid changes were restricted to a

few amino acids, there being differences between the two species in the specific compounds affected. (2) The pattern of major changes was constant within a given species.

Preliminary quantitative estimations of specific amino acids which changed were made by column chromatographic analyses of muscle samples from one toad each of each species in fresh water and 40% sea water. Ninhydrin-positive compounds found in this way are listed in Table VI. Only components attaining concentrations greater than 2 m*M*/kg. in at least one animal are listed.

TABLE VI

Major ninhydrin-positive compounds in muscles of toads

Compound (m*M*/Kg. wet wt.)	State of adaptation			
	Bufo viridis		*Bufo boreas*	
	FW	40% SW	FW	40% SW
Glycerophosphoethanolamine	2.8	3.3	1.1	4.2
Phosphoethanolamine	5.1	5.9	2.8	4.4
Taurine	19	28	8.7	23
Urea	43	84	20	59
Aspartic acid	0.3	2.5	—	—
Serine	0.6	2.6	—	—
Asparagine-glutamine	1.0	5.4	—	—
Glutamic acid	3.3	5.4	2.1	14
Glycine	2.1	12	5.0	2.7
Alanine	0.2	18.9	1.7	3.0
Creatinine	—	—	3.8	0
Carnosine	1.8	3.6	0.4	11

Considering only those compounds which changed in concentration by more than 5 m*M*/kg. wet weight, important increases occurred in *B. viridis* for taurine, urea, glycine and alanine. In *B. boreas*, similarly large increases occurred for taurine, urea, glutamic acid and carnosine. These changes were probably the only ones which were statistically significant, based on experience with column chromatographic analyses of replicate muscle samples taken from other amphibian species.

Discussion

Bufo viridis and *Bufo boreas* appear to be the first vertebrates known to use large quantities of free amino acids in intracellular osmoregulation. The hagfishes (Myxini) also have high blood salt concentrations, but no more than 10–15% of intracellular osmotic concentrations in these forms are due to free amino acids. Intracellular free amino acid concentrations in *Myxine glutinosa* in 100% sea water are only 60–70 m*M*/kg. fiber water (Bellamy and Jones, 1961). This level is about one-third that found in both species of toad adapted to only 50% sea water. Other species of toads apparently normally maintain about 100 m*M*/kg. fiber water of intracellular free amino acids, even in the absence of hydration stresses (Briner, 1961).

Free amino acids have long been known to be of importance in intracellular osmoregulation in many groups of invertebrates (recent reviews include Awapara, 1962; Florkin, 1963; Kittredge *et al.*, 1962; Lockwood, 1962).

There is as yet no experimentally defined relationship between the changes in intracellular solute composition described and the changes in intracellular osmotic concentration which occurred. Intracellular osmotic concentrations probably change with and are equal to simultaneous extracellular osmotic concentrations. While this situation has not yet been conclusively proven (Robinson, 1960, for review), data such as those of Buckley *et al.* (1958) and Bloch *et al.* (1963) seem convincing. However, all cell water may or may not be available to solutes (Dydyńska and Wilkie, 1963; Lindenberg and Gary-Bobo, 1960). Intracellular sodium activity may be quite low (Lev, 1964). Very little is known of the degree to which "free amino acids" are actually free in solution, or whether other substances, such as urea, may not actually be partially bound (Bozler, 1961; Heinz, 1962).

The differences in the nature of the compounds changing in the two species presumably are reflections of metabolic differences between the two forms. It is probable that equally large metabolic differences exist among the various tissues within each species (Roberts and Simonsen, 1962). The chemical origins of the substances changing in concentration are unknown. Changes in external ionic environment have been shown to produce important metabolic shifts in isolated muscles of *Bufo marinus* (Muller, 1962).

The change in carnosine content in *B. boreas* may be accounted for in several ways. Severin *et al.* (1962) indicate that carnosine may play a role in neuromuscular transmission in frogs. They found that the carnosine content of pieces of muscle containing many motor end plates was significantly higher than the carnosine content of pieces containing few or no end plates. The present results may thus: (1) be related to the changing needs of intracellular osmoregulation; (2) indicate an important change in neuromuscular function in *B. boreas* associated with salinity stress; or (3) be simply a sampling error due to differences in numbers of motor end plates included in the muscle samples analyzed.

The present data on muscle inulin spaces agree well with some previous determinations of this quantity in amphibian muscle (Boyle *et al.*, 1941; Dee and Kernan, 1963; Simon *et al.*, 1957). They are significantly lower and less variable than other results (Steinbach, 1961; Tasker *et al.*, 1959). These latter groups of higher and more variable results may be due to handling procedures used in experiments involving soaking of isolated muscles in Ringer solutions of various types (Dydyńska and Wilkie, 1963).

The present data on plasma composition in *B. viridis* agree with previous results (Gordon, 1962). Both sets of data are quite different from those of Tercafs and Schoffeniels (1962). The reasons for the disagreement are not apparent.

Summary

1. A detailed study of intracellular osmoregulation in skeletal muscles has been carried out in two species of toads, *Bufo viridis* and *Bufo boreas*, adapted to various external salinities between fresh water and 50% sea water (salinity 16‰).

2. Both species are osmoconformers, changes in plasma osmotic concentrations being due almost entirely to changes in NaCl concentrations. Muscle dry weight,

however, is more stable, increasing by less than 35% in both forms in the face of osmotic concentration increases of 80–135% in the plasma. Muscle extracellular volume (inulin space) is constant, independent of changes in plasma concentration.

3. Relative stability of muscle hydration is due to accumulation of intracellular solutes. Changes in intracellular osmotic concentration are broadly partitioned: 47% inorganic ions (Cl, Na, K), 33% free amino acids and related compounds, 20% urea in *B. viridis;* 43% inorganic ions, 40% free amino acids and related compounds, 17% urea in *B. boreas.* Free carbohydrates appear to be virtually absent.

4. Increases in intracellular free amino acids and related compounds involve different substances in the two species. *B. viridis* accumulates primarily taurine, glycine and alanine. *B. boreas* accumulates primarily taurine, glutamic acid and carnosine. Intracellular urea concentrations seem always to be significantly higher than plasma concentrations.

5. Uncertainties in the data, related primarily to lack of knowledge of the physical state of intracellular solutes, are discussed. Various implications of the results are considered.

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